Chemical Reaction in Tomato Plants in Response to A biotic Elicitors Treatments

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Abstract: Early blight resistant cultivar "Tezier" and susceptible cv. "Castle rock" were tested to identification their response to *A. solani* infection on tomato seedlings pre-treated with chemical inducers: Salicylic acid (SA), Isonicotinic acid (INA), and Thiamine (vit. B_1), under greenhouse conditions. Resistant cv. "Tezier" exhibited rapid reaction represented in higher significant endogenous SA levels compared to the susceptible cv. "Castle rock" for all chemical treatments. "Tezier" endogenous SA levels surpassed "Castle Rock", 5 folds in exogenous SA, 2 fold in INA, and about 5 folds for vit. B_1 application. "Tezier" also had higher quantities in PRs accumulation (β-1, 3-glucanase, chitinase and peroxidase) in time course intervals 3, 24, 48, 72, and 96 hrs after pathogen inoculation, through increase of PRs activity which was started one day after inoculation in all the induced plants and reached maximum level after three to four days compared to "Castle rock" for all chemical inducers. Total protein content and polyphenol oxidase activity were also observed, their levels were highly significant in "Tezier".
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1. Introduction:

Infection of plants with a necrotizing pathogen can enhance resistance to subsequent infections by various fungal, bacterial and viral pathogens. This induced resistance, known as systemic acquired resistance (SAR), extends to plant tissue distant from the infection site and can persist for weeks after the initial infection. Salicylic acid (SA) plays an important role in signal transduction in plants and is believed to initiate SAR (Malamy *et al.*, 1990). Peng *et al.*, (2004) indicate that the SA pathway is involved in a wide range of plant defense responses, and SA is a key regulator of pathogen-induced systemic acquired resistance. SA has also been found to activate the expression of genes that encode pathogenesis-related proteins (Yalpani *et al.*, 1991). Exogenous application of SA to roots of hydroponically grown tomato can increase resistance against *A. solani*, the causal agent of early blight (Spletzer and Enyedi, 1999).

Pathogenesis-related proteins (PRs), identified as inducible proteins that have been implicated in active defense and could play a role in restricting pathogen development and spread in the plant against various pathogens (Chen and Zhu 2004; Eulgem, 2005). The recognized PRs have been extensively reviewed and currently comprise 17 families of induced proteins (van Loon *et al.* 2006). Chitinases and β-1,3-glucanases catalyze the hydrolysis of chitin and β-1,3-glucan, respectively, both polymers major components of the fungi cell walls. Typically they are expressed constitutively at low levels in plant cell and accumulate in response to fungal, bacterial, viral attack, or other inducers of acquired resistance (Gunter *et al.*, 2008; Cota *et al.* 2006). Time-course accumulation of chitinase and β -1,3-glucanase in induced plants was significantly higher than the control. Maximum activities of these PR-proteins were recorded after three days of inoculation in all induced plants. Thereafter, the activity decreased progressively (Saikia *et al.* 2005). Polyphenoloxidase (PPO) the oxidative enzyme converts phenolic compounds of plants into polyphenols and quinones, the toxic substances for theextracellular enzymes produced by the pathogens (Raju *et al.*, 2008). Peroxidase (POD) is a key enzyme in thebiosynthesis of lignin, where lignification leading to disease resistance (Bruce and West, 1989). The late and generalized necrosis in the susceptible cultivars seems to be related to the intervention of the PPO, contrary to the resistant cultivars in which the fast and localized induction of necrosis was associated to the POD (Diani *et al.* 2009). Specific chemicals such as salicylic acid (SA), benzo[1,2,3]thiadiazole-7-carbothionic acid-S-methyl ester (BTH, also named acibenzolar-Smethyl), and dl-3-amino-n-butyric acid (BABA) have been reported to induce SAR in a variety of plants against

a wide range of microbial pathogens without possessing direct antimicrobial activity (Barilli *et al.* 2010).

The objective of this study was to determine differences between resistant and susceptible cultivars in rate of induction of SA expression and monitoring accumulation of PRs during time intervals due to the induction by certain chemical activators.

2. Materials and methods

Tomato (*Lycopersicon esculentum* Mill) cultivars Tezier (resistant) and Castel rock (susceptible) were grown under greenhouse conditions (24-26 °C) in 14 cm pots containing : mixture of 1:2:1 sand: clay: peatmoos planted at the rate of 5 seedlings per pot. For seedlings inoculation, conidial obtained from (*Alternaria solani*, virulent isolate, obtained from Mycological center Assiut University).

Ellis and Martin) culture grown on potato dextrose agar for 10 days according to the method described by El-Samra *et al.* (2009). Eight tomato plants (5-week-old) of each cultivar were sprayed with Salicylic acid (SA) 500 ppm, Isonicotinic acid (INA) 750 µl/L, and Thiamine (vit. B_1) 100 mM on the upper and lower leaf surface which were solubilizing in water or ethyl alcohol. As a control treatment, H_2O was used instead of chemical inducer solution in each case. The same tomato seedlings pretreated by chemical inducers were inoculated by spraying with a suspension of 1×10^4 conidia ml^{-1}. Inoculated plants were incubated at 100% RH by covering bots with plastic bags for 24 h. Individual leaves: at each sampling time; 3, 24, 48, 72, 96 hrs after fungal inoculation to account for variation in SA content and PR protein levels were collected and pooled in liquid nitrogen for further studies.

Chemicals

Salicylic acid (SA) Sigma-Aldrich Chemie-France**,** Isonicotinic acid (INA), Vitamin B_1 hydrochloride (vit.B_1), were purchased from Sigma-Aldrich chemicals company (Cairo). Other chemicals and solvents used in this work were analytical or chromatographic grade. A standard HPLC calibration solution of salicylic acid, concentrations; 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 µg were prepared by accurate step-by-step dilutions of stock solution 10 µg by weighing 1 g SA and dissolving in 100 ml methanol.

Quantification of free salicylic acid (SA):

Quantification of endogenous SA was carried out twice, the first after 7 days from chemical application and the second after 7 days from pathogen inoculation on the resistant and susceptible tomato cultivars. Salicylic acid levels were measured in leaf tissue by high performance liquid chromatography (HPLC).

Extraction of free SA

Free SA was extracted from tomato plants according to the method of Malamay and Klessig (1992) with some start time after 3 hours of fungal inoculation.

Determination of SA:

Leaf material was grounded without sand particles, Supernatant evaporation was carried out by heating at 40 °C for 24 hours, were analyzed by HPLC-electrospray ionisation using an Agilent 1100 HPLC coupled to an Applied Biosystems Q-TRAP 2000 (Applied Biosystems, California, USA). Chromatographic separation was carried out on a Phenomenex Luna 3 µm C18 (2) 100 mm × 2.0 mm column, at 35°C. Determination of endogenous SA levels was performed according to the method of Forcat *et al.*, (2008).

Preparation of leaf homogenates

For determining enzyme activities of peroxidase (PO), and polyphenoloxidase (PPO), entire leaves, collected at different time intervals (3, 24, 48, 72, 96 hrs) following inoculation, immersed in liquid N2 and homogenized with 0.1 M Na-acetate buffer (pH 5.2) (1 g plant material in 10 ml). The homogenated leaves were centrifuged at 15000 rpm for 30 min at 4 ˚C, and the enzyme activities were determined in the supernatants. For determining pathogenesis related (PR) proteins (chitinase, β -1,3-glucanase), detached leaves were immersed in liquid N_2 then homogenized in 2 ml 0.1 M Na-acetate buffer of pH 5.2 consisting of 1% (v/v) PVPP (polyvinylpolypyrolidone), 5% (v/v) glycerol, 0.1 M phenylmethansulfanylfluorid, and 0.1 M DTT (dithiothreitol). Homogenates were centrifuged at 15000 rpm for 30 min at 4 ˚C. Supernatants were used to determine enzymatic activities.

Determination of protein

Protein concentration was determined for all experiments using the method described by Bradford (1976) spectrophotometrically at 595 nm using bovine serum albumin (BSA) (0-5.0 mg/ml) as standard.

Determination of enzyme activities Peroxidase (PO) activity

Peroxidase activity was determined spectrophotometrically using guaiacol as a common substrate for peroxidases as described by Abdal Razik *et al.* (2008). Peroxidase activity = OD436nm / mg protein.

Polyphenoloxidase (PPO) activity

Peroxidase activity was determined using the method of Batra and Kuhn (1975). PPO units = OD410nm / mg protein.

Chitinase activity

For chitinase assay, the substrate colloidal chitin was prepared from chitin powder according to

the method described by Ried and Ogrud-Ziak (1981). Reducing sugars were determined in 1 ml of the supernatant by dinitrosalicylic acid (Monreal and Reese, 1969) using 1 ml of 1% colloidal chitin in 0.05 M citrate phosphate buffer (pH 6.6) in test tubes, 1 ml of enzyme extract was added and mixed by shaking. Tubes were kept in water bath at 37˚C for 60 minutes, then cooled and centrifuged before measuring O.D. at 540 nm. Chitinase activity was defined as mM N-acetylglucose amine equivalent released/gram fresh weight tissue/60 minutes.

β-1,3-glucanase activity

Total β-1,3-glucanase activity was colorimetrically assayed by the laminaria-dinitrosalicyclate method described by Saikia *et al.* (2005). One gram of tomato leaves was extracted with 5 ml sodium acetate buffer (SAB; 0.05 M), pH 5. The extract was then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was then used as crude enzyme extract. The extract (62.5 µl) was added to laminarin (4%, 62.5 µl) and incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic acid reagent and heated for 5 min in boiling water bath. The resulting coloured solution was diluted with 4.5 ml water, vortexed and absorbance at 500 nm was determined. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as µmol equivalent glucose release min^{-1} g^{-1} fresh tissue.

Statistical analysis

Salicylic acid data were compared using Scheffe's test ($P \leq 0.05$). Antifungal activity as well as chitinase, β-1,3-glucanase, polyphenoloxidase and peroxidase activity were statistically analyzed by Fisher's LSD Test ($P \leq 0.05$) (Gomez and Gomez, 1984). The package used for analysis was NCSS and PASS software version 2000.

3. Results:

SA expression in 'Resistant' and 'Susceptible tomato cvs.

A. Seven days after chemical treatment:

Data in Table (1) and Fig. (1) Showed no salicylic acid was detected in untreated control of resistant and susceptible cultivars. Moreover, in general SA content in resistant cultivar was in general SA content in resistant cultivar was significantly higher than those of susceptible ones. The highest SA contents 3.36 µg/$^{g-1}$ fresh weight, was obtained in Tezier cv., treated with thiamine, compared with that of the same treatment in Castle Rock cv. , 0.15 µg/g^{-1} FW SA contents in resistant cv., treated with SA or INA were recorded as 0.19 and 0.66 µg/g^{-1} FW, respectively. Unlike the resistant cv. treatments, the highest SA content in susceptible cultivar, 0.21 µg/g^{-1} FW, was obtained in seedlings, treated with INA, followed by those of SA and thiamine treatments as 0.03 and 0.15 µg/g^{-1} FW respectively

B. Seven days after inoculation with *A.solani*:

Similar trend was also observed concerning the susceptible cultivar Castle Rock.

Salicylic acid content in non-inoculated untreated control of resistant cv. (0.27 µg/g^{-1} FW) was higher than that of susceptible cultivar (0.04 µg/g^{-1} FW). Moreover, SA levels in all inoculated resistant treatments were, generally, significantly higher than those of the susceptible ones (Table 1 and Fig. 1). The highest SA content (0.34 µg/g^{-1} fresh weight) was obtained in inoculated Tezier cv., treated with thiamine, compared with that of the same treatment in Castle Rock cv. (0.07 µg/g^{-1} FW)**.** SA contents in inoculated resistant cv., treated with SA or INA (0.05 and 0.12 µg/g^{-}1 FW, respectively) were less than that of thiamine treatment (0.34µg/g^{-1} FW). Table (1). SA concentration (µg/g^{-1}) Contents of SA were estimated after 7 days both of chemical or fungal treatments days after inoculation with *A.solani*, under greenhouse conditions . Data in Table (1) and Fig. (1).

Pathogenesis related proteins (PRs)

Total protein determination

Protein content was determined by the method of Bradford (1976), with bovine serum albumin as the standard.

Chitinase activity

A standard curve was established for subsequent determination of induced chitinase in the tested tomato plants by resulted N-acetylglucosamine (mM per one gram fresh weight). Generally, untreated control inoculated with *A.solani* (C2) showed significant increase in chitinase activity in both resistant Tezier and susceptible Castle Rock cvs., compared with that of untreated non-inoculated control (C1). All the tested chemical inducers of SAR against *A.solani* significantly induced chitinases activity both in resistant and susceptible cvs., compared with both of untreated inoculated and non-inoculated control (C1 &C2). However, increasing rates significantly differed according to the applied chemical inducer, host resistance and time elapsed after inoculation with *A.solani*. Resistant Tezier cultivar, activity of chitinases, induced by SA increased gradually with time elapsed after inoculation, attaining maximum rates at the end of the experiment (14.68 and 12.98 and 6.96 times over that of C2 control, respectively). Meanwhile, INA and vit.B_1, increased chitinases activity gradually up to 48 hrs then decreased.

As for susceptible Castle Rock cv., treatment with vit.B_1 resulted in the highest increase in chitinases activity after three hours of treatment

(11.62-fold Moreover, the highest induction rate was realized by vit. B1 (1.77-fold), compared with SA and INA (1.15 and 1.19- fold of untreated inoculated control, respectively). Generally, vit. And SA were more efficient in inducing enzyme activity than INA. Pronounced decrease in enzyme activity was realized at the end of the experiment (0.29% of that of C2), start time was after 3 hours of fungal inoculation.

Treatment with vit.B_1 resulted in the highest increase in chitinases activity after three hours of inoculation (11.62-fold of C_2, compared with SA and INA (5.11 and 1.55-fold, respectively). The highest induction rate was realized by vit.B_1 (14.137 mM/g^{-1} FW), compared with SA and INA (9.189 and 9.521 mM/g^{-1} FW, respectively, after 48 hours of inoculation). Generally, vit.B_1 and SA were more efficient in inducing enzyme activity than INA. Pronounced decrease in enzyme activity was realized at the end of the experiment (0.29% of that of C_2 in INA treatment).

β-1, 3-Glucanase activity

Standard curve was established for subsequent determination of induced β -1,3-Glucanase in the tested tomato plants by resulted glucose (µmol per one gram fresh weight).

According to the results shown in Table 3 and illustrated in figs, 4 and 5, all the tested chemical inducers of SAR against *A.solani* significantly induced β -1,3-Glucanase activity both in resistant and susceptible cvs., compared with that of untreated non-inoculated control C1 and most of untreated inoculated control C2. However, increasing rates significantly differed according to the applied chemical inducer, host resistance and time elapsed after inoculation with *A.solani*.

In resistant Tezier cultivar, activity of β -1,3-Glucanase, induced by SA, INA and vit.B_1 increased gradually with time elapsed after inoculation, attaining maximum rates 48 hours after inoculation (6.009, 5.962 and 5.747 µmol /g^{-1} FW, respectively), then gradually decreased until the end of experiment. The highest enzyme activity values 96 hrs. After inoculation was detected in vit.B_1 treatment. Although enzyme activities induced by the tested inducers were higher than those induced by inoculated untreated control, however, differences were mostly limited (Table 3 and Fig.4).

In susceptible Castle Rock cv., treatment with SA, INA and vit.B_1 resulted in significant increase in β -1,3-Glucanase directly after inoculation with *A.solani* (54.68, 54.54 and 55.11%, respectively more than that of C1). Enzyme activity in treated inoculated plants increase with time elapsed after inoculation, attaining relatively highest levels after 48 hours, and then showed slight gradual decrease until the end of the experiment. Increases in enzyme activities, observed in inoculated untreated control C2 along the experiment were in close similarity to those induced by all the tested inducers at all the tested time intervals after inoculation, however, activities in C2 surpassed those induced by SA and vit.B_1. Generally, differences in enzyme activities among inducer treatment and those of C2 were not pronounced in the susceptible cultivar, compared with those of the resistant cv. (table 3 and fig. 5).

Data in table 3, illustrated in figs. 4 and 5 showed pronounced increase in â-1,3-Glucanase between resistant Tezier cv. and susceptible Castle Rock cv.. in particular 48 hours after inoculation of tomato plants, pretreated with SA, INA and vit.B_1 with *A.solani* (12, 12.07 and 7.64%, respectively, more than those of susceptible cv.). At the fourth day after inoculation,, treatment of resistant cv. with vit.B_1 exhibited 5,66% increase in enzyme activity, compared with that of susceptible cv.

Peroxidase (PO) activity

In resistant Tezier tomato cv., it was detected that inoculation of untreated plants with *A.solani* resulted in significant induction of peroxidase activity, compared with that of C1 control. Moreover, enzyme activity increased with time elapsed after inoculation, attaining highest values after 48 hours (4-fold over that of C1 control). Gradual decrease was the occurred until the end of the experiment, where enzyme activity in C2 control was 2.65-fold over that of C1 control.

According to results of (Table 4, Fig. 6), treatment with any of the tested SAR chemical inducers resulted in significant increase in enzyme activity, compared with that of the untreated inoculated C2 control. However increasing rates differed according the tested inducers. Three hours after inoculation significant increase in enzyme activity, in particular those pretreated with SA and INA (2.54 and 2.15-fold, respectively over that of C2 control). The highest levels of peroxidase activity occurred 48 hours after inoculation in plants pretreated with any of the tested inducers, however INA was more active in this respect (2-fold of C2 control), followed by vit. B_1 (1.3-fold of C2 control).

At the end of the experiment, increasing rates in enzyme activity were, generally lower than the other tested intervals, however INA and SA treatments realized, relatively higher rates (about 1.6-fold of that of C2). M β

Generally, INA was the most effective in inducing peroxidase activities after the second day of inoculation until the end of the experiment, followed by SA

In susceptible Castle Rock cv., it was evident that inoculation of untreated susceptible cv.

with *A.solani* resulted in significant increase in PO activity directly after three hours and 24 hours (3.68 and 3.8-fold over that of untreated non-inoculated C1 control), attaining maximum values at the end of the experiment 96 hours after inoculation, where PO activity was 6.18-fold over that of C1 control.

Results in table 4 and Fig. 7 showed that pretreatment of susceptible plants with the tested SAR chemical inducers, i.e. SA, INA and vit.B_1 before inoculation with *A.solani* significantly increased PO activity mostly from the first day of inoculation and till the end of the experiment. However, increasing rates, compared with PO activity in C2 control significantly differed according to the tested inducers and time elapsed after inoculation.

Three hours after inoculation, significant increase in PO activity was detected in both SA and vit.B_1 treatments (9% and 24.73%, respectively more than that of C2 control). After 48 hours of inoculation, INA exhibited the highest values of PO activity (2.36-fold over that of C2 control), whereas SA showed the highest PO activity after 72 hours (2.22-fold of C2 control). PO activity was then decreased until the end of the experiment.

Generally, INA and SA increased more PO activity particularly after the second and third days of inoculation.

Polyphenoloxidase (PPO) activity

In resistant Tezier cv., inoculation of untreated plants (Table 5) with *A.solani* significantly increased PPO activity throughout the duration of the experiment, attaining maximum activity (0.352 units/mg protein) at the end of the experiment (1.68-fold over that of C1). Treatment resistant tomato plants with the tested chemical inducers significantly increased PPO activity, compared with C2, however, significant decrease in enzyme activity at the end of the experiment, compared with that of the untreated inoculated control (C2).

Increasing rates differed according to the tested inducer and time after inoculation with *A.solani* (Table 5). A pronounced increase in PPO activity was detected (Table 5) in plants pretreated with vit.B_1 directly after inoculation (73.22% more than C2 control), attaining highest PPO activities after 48 hours (0.802 units/mg protein), compared with those induced by SA and INA inducers (0.572 and 0.514 units/mg protein, respectively). Gradual decrease in PPO activity was observed at the end of the experiment, where PPO activity values were less than that of C2 control. Generally, vit.B_1 was more efficient in inducing PPO activities in resistant cv. inoculated with *A.solani* than the other tested inducers, followed by SA (Fig. 8).

In susceptible Castle Rock cv., inoculation of untreated plants with *A.solani* resulted in an increase in PPO activity over that of C1 control, attaining maximum value 48 hours after inoculation (0.165 units/mg protein) then gradually decreased to reach the minimum enzyme activity at the end of the experiment (0.087 units/mg protein) (Table 5). Pretreatment of the susceptible Castle Rock cv. with the chemical SAR inducers INA and vit.B_1 significantly induced an increase of PPO activity, attaining maximum values two days after inoculation (0.241 and 0.211 units/mg protein, i.e. 31.54% and 21.80%, respectively than that of C2 control) (Table 5). Although treatment with SA resulted in the highest PPO activities (45.20% more than C2 control), three hours after inoculation, however, it decreased, attaining values less than that of C2 control at both the second and the third days of inoculation. Generally, treatment of the susceptible cv. with INA realized the highest induction of PPO, at the second day of inoculation with *A.solani*, followed by vit.B_1 (Fig. 9).

4. Discussion

Plant pathogen interactions are rapid and dynamic, with both host and pathogen constantly wrestling to modify signaling networks and reconfigure metabolism in favor of defense or disease (Truman *et al.* 2010). SA accumulates in leaf tissue following infection by an avirulent pathogen, but SA levels have not been reported in susceptible cultivars and compared to resistant ones. Though SA accumulation in susceptible cultivars has not been measured, a susceptible cultivar of tomato has the ability to take up exogenous SA (Spletzer and Enyedi, 1999), INA, and vit. B_1 and express resistance to *A.solani*. The link between a SA response and a hypersensitive reaction (Conrath *et al.*, 1995) suggests that resistant cultivars can accumulate SA more quickly than susceptible cultivars. In a test of this hypothesis, early blight resistant cv. 'Tezier' showed a more rapid accumulation of SA than 'Castle Rock'. This supports the hypothesis that faster accumulation of SA upon pathogen recognition occurs in resistant cultivars than in susceptible cultivars. In these experiments, we demonstrated that SA can activate a form of systemic resistance against *A. solani* in tomato plants grown under greenhouse conditions. This was accomplished by providing 500 ppm SA, 750ml/L INA, and 100 mM vit. B_1, directly foliar application.

Table (1). Contents of SA (µg/g^{-1}) in resistant (Tezier) and susceptible (Castle Rock) tomato cultivars pretreated with abiotic inducers under greenhouse conditions.

cultivar	treatment	SA content µg/g^{-1} FW	
		7 days after inducer application	7 days after pathogen inoculation
Tezier	Control (H_2O)	0.00^{a}	0.27^{a}
	SA	0.19^{c}	0.05^{b}
	INA	0.66^{d}	0.12^{c}
	vit. B_1	3.36^{e}	0.34^{d}
Castle Rock	Control (H_2O)	0.00^{a}	$0.04b^{e}$
	SA	0.03^{a}	0.01^{e}
	INA	0.21^{c}	0.06^{b}
	vit. B_1	0.15^{b}	0.07^{b}

Values followed by the same letter(s) in each column don't differ significantly according to Scheffe's test ($P \leq 0.05$)

Table (2). Chitinase activities in early blight resistant and susceptible tomato cvs. pretreated with the tested chemical SAR inducers and inoculated with *A. solani* at different periods following inoculation.

Enzyme activity (mM/g^{-1} FW)							
		Time after inoculation (hrs)					*FLSD*
Cultivars	Treatments	3	24	48	72	96	(0.05)
Tezier	C1	0.623^{a*}	1.205^{a*}	1.839^{a}	1.890^{a}	3.056^{a*}	0.159
(resistant)	C2	1.164^{b}	2.39^{b*}	12.205^{b*}	10.638^{b*}	1.101^{b}	0.180
	SA	9.106^{c*}	11.975^{c*}	14.158^{c*}	15.01^{c*}	16.174^{c*}	0.824
	INA	2.723^{d*}	3.201^{d*}	17.588^{d*}	11.808^{d*}	6.759^{d*}	0.208
	Vit.B_1	8.170^{e}	8.503^{e}	14.49^{e*}	12.91^{e}	13.097^{e}	0.826
Castle Rock	C1	0.528^{f}	0.645^{f}	1.210^{f*}	0.989^{f*}	0.542^{f}	0.160
(susceptible)	C2	0.696^{g*}	1.538^{g*}	7.995^{g*}	5.667^{g*}	7.563^{g*}	0.179
	SA	3.555^{h*}	9.272^{h}	9.189^{h}	10.893^{h*}	13.617^{h*}	0.181
	INA	1.081^{i*}	2.245^{i}	9.521^{i*}	9.771^{i*}	2.162^{i}	0.162
	Vit.B_1	8.087^{k*}	8.648^{k*}	14.137^{k*}	11.101^{k*}	12.203^{k*}	0.139
FLSD (0.05)		0.050	0.069	0.020	0.074	0.030	

Values followed by the same letter(s) in each column don't differ significantly according to Fisher's LSD Test ($P \leq 0.05$).
Values with superscript star (*) differs significantly than other values in each row according to Fisher's LSD Test ($P \leq 0.05$).
C1 = untreated non-inoculated control, C2 = untreated inoculated control.

Table (3). β-1,3-Glucanase activities in early blight resistant and susceptible tomato cvs. Pre-treated with the tested chemical SAR inducers and inoculated with *A.solani* at different periods following inoculation.

Enzyme activity (µmol/g^{-1} FW)							
		Time after inoculation (hrs)					*FLSD*
Cultivars	Treatments	3	24	48	72	96	(0.05)
	C1	1.682^{a*}	2.663^{a}	2.616^{a}	1.915^{a*}	2.757^{a*}	0.080
Tezier	C2	4.878^{b}	4.785^{b}	4.588^{b}	4.785^{bd}	4.616^{cd}	0.113
(resistant)	SA	4.523^{cg}	5.242^{bc*}	6.009^{c*}	5.130^{dg*}	4.523^{c}	0.083
	INA	4.869^{b}	5.009^{bd}	5.962^{c*}	4.373^{b}	4.766^{df}	0.815
	Vit.B_1	4.747^{be}	5.439^{cd}	5.747^{c}	5.000^{cde}	5.121^{g}	0.915
Castle Rock	C1	2.766^{d*}	1.897^{e*}	2.383^{a}	2.467^{a}	2.252^{b}	0.199
(susceptible)	C2	4.626^{ce}	4.551^{b}	5.411^{c}	5.355^{efgh}	5.177^{g}	0.214
	SA	4.467^{cf}	4.850^{bc}	5.280^{bc}	5.074^{cdf}	4.738^{de}	0.827
	INA	4.728^{be}	4.672^{b}	5.242^{bc}	5.785^{h*}	5.289^{g}	0.198
	Vit.B_1	4.383^{fg}	4.878^{bc}	5.308^{bc*}	4.504^{bc}	4.831^{ef}	0.200
FLSD (0.05)		0.160	0.488	0.780	0.582	0.178	

Values followed by the same letter(s) in each column don't differ significantly according to Fisher's LSD Test ($P \leq 0.05$).
Values with superscript star (*) differs significantly than other values in each row according to Fisher's LSD Test ($P \leq 0.05$).
C1 = untreated non-inoculated control, C2 = untreated inoculated control.

Table (4). Peroxidase (PO) activities in early blight resistant and susceptible tomato cvs. pre-treated with the tested chemical SAR inducers and inoculated with *A.solani* at different periods following inoculation.

Enzyme activity (units/mg protein)							
Cultivars		Time after inoculation (hrs)					*FLSD*
	Treatments	3	24	48	72	96	(0.05)
Tezier (resistant)	C1	0.001^{a}	0.022^{a}	0.075^{a}	0.098^{a}	0.070^{a}	0.037
	C2	0.054^{b}	0.082^{b}	0.302^{b}	0.206^{b}	0.186^{b}	0.113
	SA	0.137^{c}	0.285^{c}	0.333^{c}	0.306^{c}	0.295^{c}	0.181
	INA	0.116^{d}	0.221^{d}	0.601^{d*}	0.338^{d}	0.298^{d}	0.117
	Vit.B_1	0.054^{b}	0.097^{b}	0.390^{e}	0.221^{e}	0.217^{e}	0.199
Castle Rock (susceptible)	C1	0.019^{e}	0.021^{a}	0.072^{f*}	0.032^{f}	0.017^{f}	0.077
	C2	0.070^{f}	0.080^{e}	0.167^{g}	0.121^{g}	0.105^{g}	0.140
	SA	0.077^{g}	0.199^{f}	0.289^{h}	0.269^{h}	0.201^{h}	0.293
	INA	0.055^{b}	0.096^{f}	0.394^{i}	0.199^{i}	0.136^{i}	0.269
	Vit.B_1	0.093^{h}	0.101^{h}	0.153^{k}	0.145^{k}	0.128^{k}	0.162
FLSD (0.05)		0.001	0.001	0.002	0.005	0.002	

Values followed by the same letter(s) in each column don't differ significantly according to Fisher's LSD Test ($P \leq 0.05$).
Values with superscript star (*) differs significantly than other values in each row according to Fisher's LSD Test ($P \leq 0.05$).
C1 = untreated non-inoculated control.
C2 = untreated inoculated control.

Table (5). Polyphenoloxidase (PPO) activities in early blight resistant and susceptible tomato cvs. Pre-treated with the tested chemical SAR inducers and inoculated with *A.solani* at different periods following inoculation.

Enzyme activity (units/mg protein)							
		Time after inoculation (hrs)					*FLSD*
Cultivars	Treatments	3	24	48	72	96	(0.05)
Tezier (resistant)	C1	0.131^{a}	0.180^{a}	0.201^{a}	0.198^{a}	0.210^{a}	0.269
	C2	0.169^{b}	0.259^{b}	0.281^{b}	0.209^{b}	0.352^{b}	0.304
	SA	0.211^{c}	0.498^{c}	0.572^{c}	0.293^{c}	0.231^{c}	0.128
	INA	0.437^{d}	0.366^{d}	0.514^{d}	0.339^{d}	0.350^{ad}	0.115
	Vit.B_1	0.631^{e}	0.662^{e}	0.802^{e}	0.395^{e}	0.283^{e}	0.140
Castle Rock (susceptible)	C1	0.070^{f}	0.099^{f}	0.101^{f}	0.122^{f}	0.052^{d}	0.121
	C2	0.097^{g}	0.146^{g}	0.165^{g}	0.150^{g}	0.087^{f}	0.116
	SA	0.177^{h}	0.195^{h}	0.138^{h}	0.136^{h}	0.118^{g}	0.081
	INA	0.121^{i}	0.176^{a}	0.241^{i}	0.218^{i}	0.150^{h}	0.082
	Vit.B_1	0.160^{k}	0.218^{k}	0.211^{a}	0.154^{g}	0.138^{i}	0.182
FLSD (0.05)		0.006	0.012	0.022	0.003	0.007	

Values followed by the same letter(s) in each column don't differ significantly according to Fisher's LSD Test ($P \leq 0.05$), C1 = untreated non-inoculated control, C2 = untreated inoculated control.

In each experiment performed, there was a significant decrease in when the pathogen inoculation was preceded by SA, INA, and vit. B_1 treatments. These chemicals are known to be a potent inducers of systemic resistance in many plants including; tobacco, cucumber, potato, and Arabidopsis (White 1979; Ward *et al.* 1991; Coquoz *et al.*, 1995; Dann and Deverall 1995; Lund *et al.*, 1998; Dong and Beer, 2000; and Ahn *et al.* 2005) consequently, it is not surprising that these chemicals exerts a similar effect in tomato. In tomato, SAR can be induced using several different biotic elicitors; earlier work has shown that inoculation with *P. infestans* (Enkerli *et al.*, 1993 and Heller and Gessler 1986), tobacco necrosis virus (Anfoka and Buchenauer, 1997) and the host-incompatible *Meloidogyne incognita* nematode (Ogallo and McClure 1996) all induce SAR in tomato.

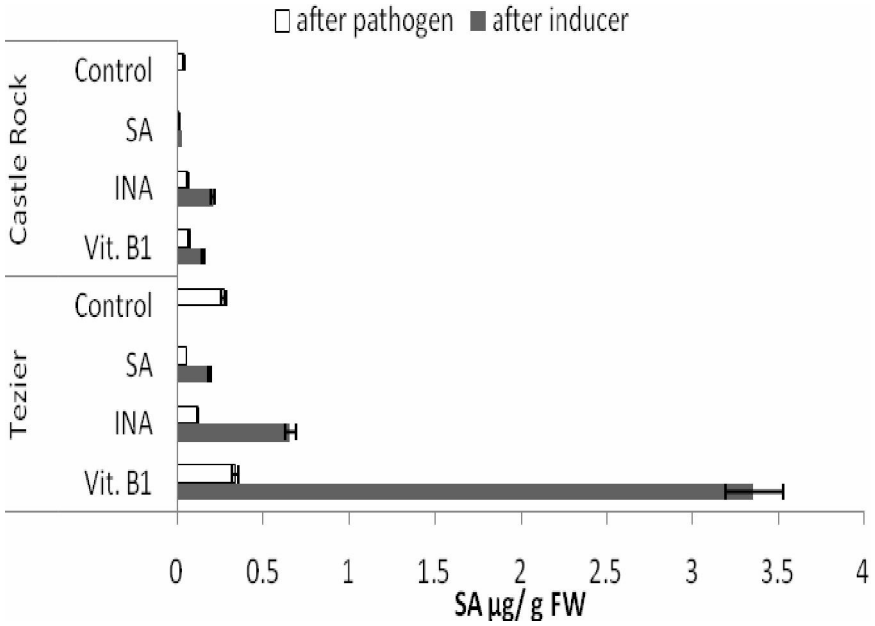

Fig.1. Endogenous levels of free salicylic acid (SA) in resistant cv. Tezier and susceptible cv. Castle Rock after inducer application (shaded bars) and after pathogen (*A. solani*) inoculation (open bars) on tomato plants. Leaves were harvested 7 and 14 days respectively, after treatment with H_2O (control) or 500 ppm SA, 750 ml/L INA, and 100 mM vit. B_1. Data bars are the means (± standard error) of three replicates.

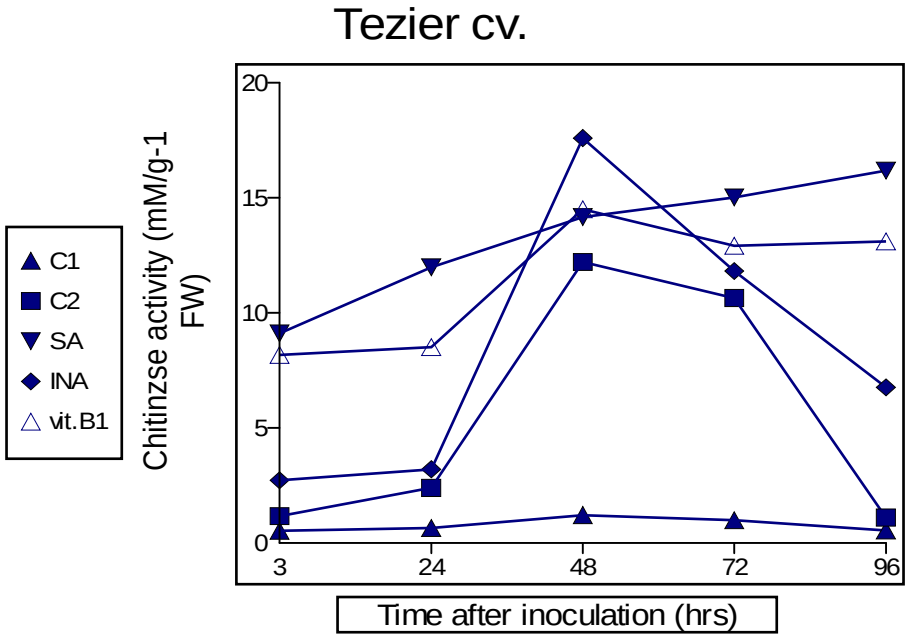

Fig. (2). Time course of chitinase activities in early blight resistant tomato cv., pre-treated with the tested chemical SAR inducers and inoculated with *A.solani*, at different periods following inoculation. C1 = untreated non-inoculated control, C2 = untreated inoculated control.

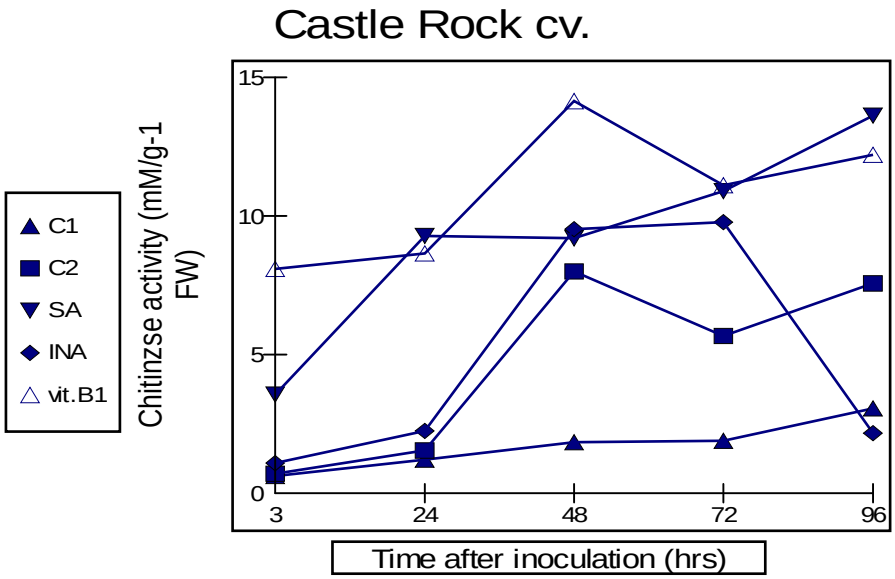

Fig. (3). Time course of chitinase activities in early blight susceptible tomato cv., pre-treated with the tested chemical SAR inducers and inoculated with *A.solani*, at different periods following inoculation. C1 = untreated non-inoculated control, C2 = untreated inoculated control.

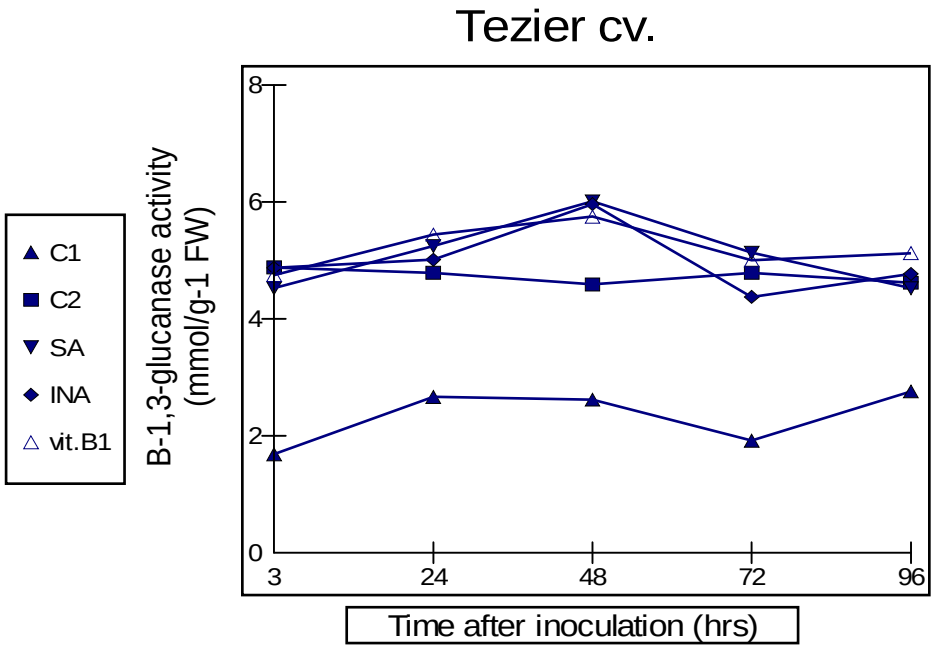

Fig. (4). Time course of β-1,3-glucanase activities in early blight resistant tomato cv., pre-treated with the tested chemical SAR inducers and inoculated with *A.solani*, at different periods following inoculation. C1 = untreated non-inoculated control, C2 = untreated inoculated control.

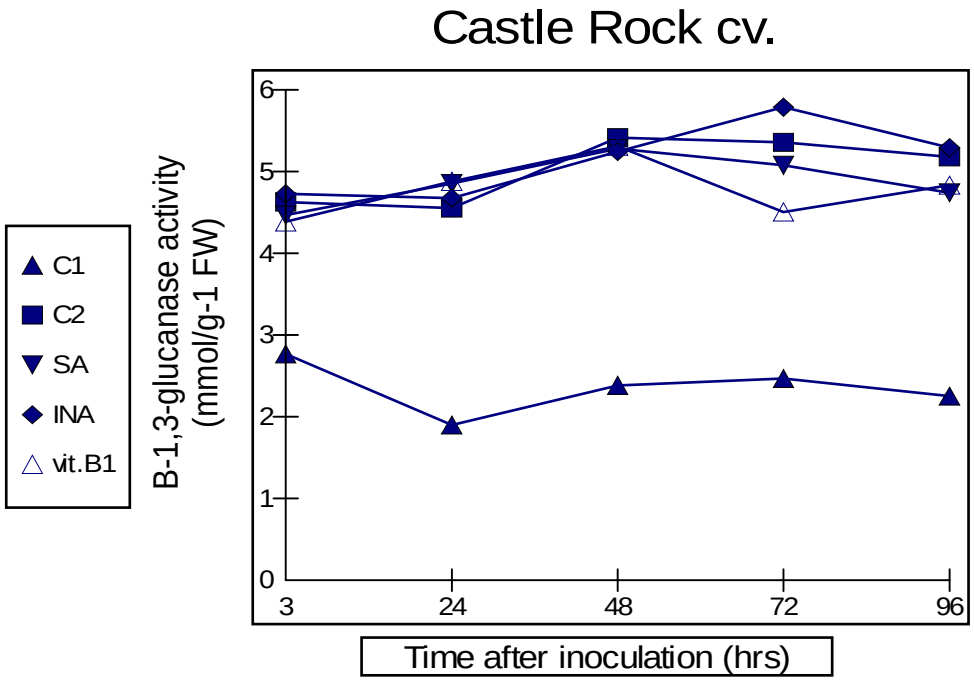

Fig. (5). Time course of β-1,3-glucanase activities in early blight susceptible tomato cv., pre-treated with the tested chemical SAR inducers and inoculated with *A.solani*, at different periods following inoculation. C1 = untreated non-inoculated control, C2 = untreated inoculated control.

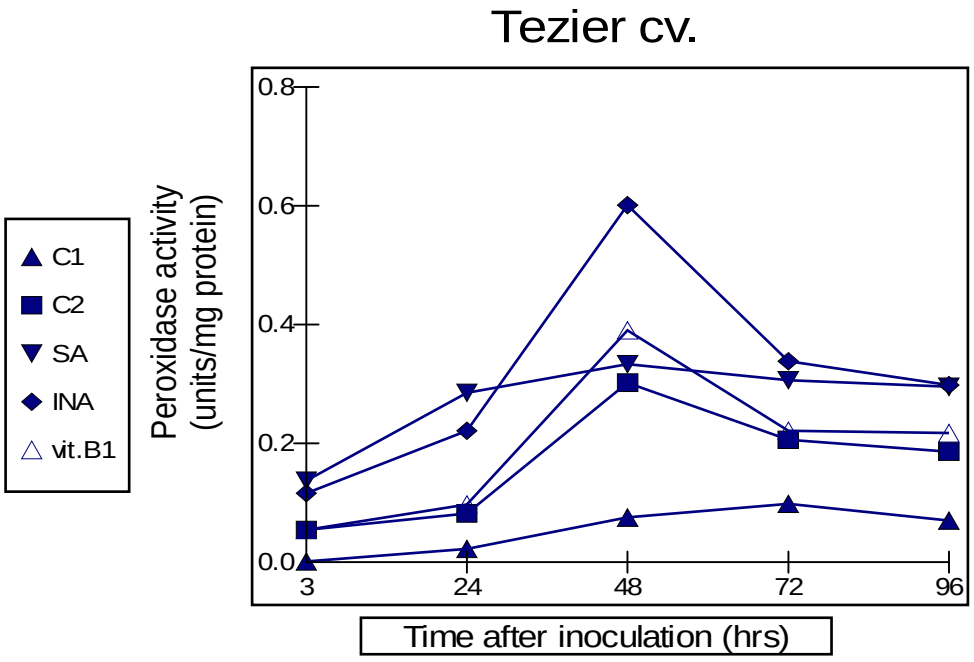

Fig. (6). Time course of peroxidase activities in early blight resistant tomato cv., pre-treated with the tested chemical SAR inducers and inoculated with A.solani, at different periods following inoculation. C1 = untreated non-inoculated control, C2 = untreated inoculated control.

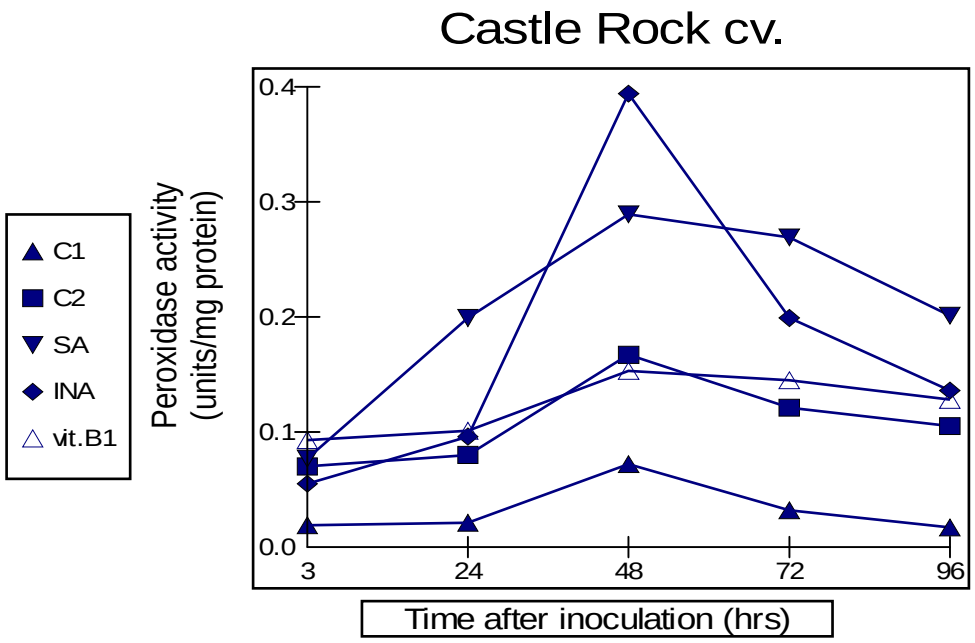

Fig. (7). Time course of peroxidase activities in early blight susceptible tomato cv., pre-treated with the tested chemical SAR inducers and inoculated with A.solani, at different periods following inoculation. C1 = untreated non-inoculated control, C2 = untreated inoculated control.

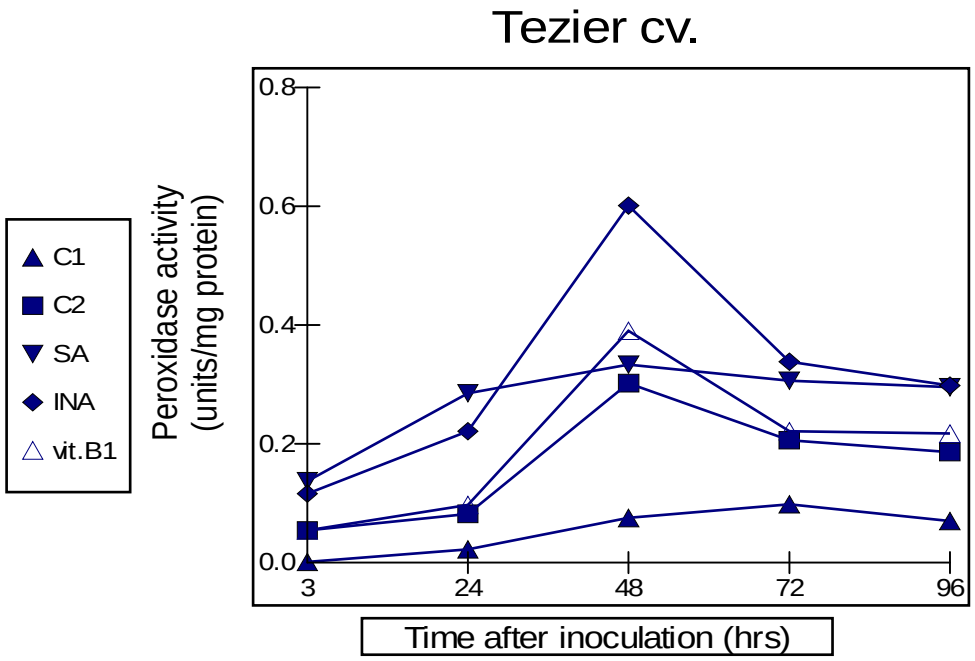

Fig. (6). Time course of peroxidase activities in early blight resistant tomato cv., pre-treated with the tested chemical SAR inducers and inoculated with A.solani, at different periods following inoculation. C1 = untreated non-inoculated control, C2 = untreated inoculated control.

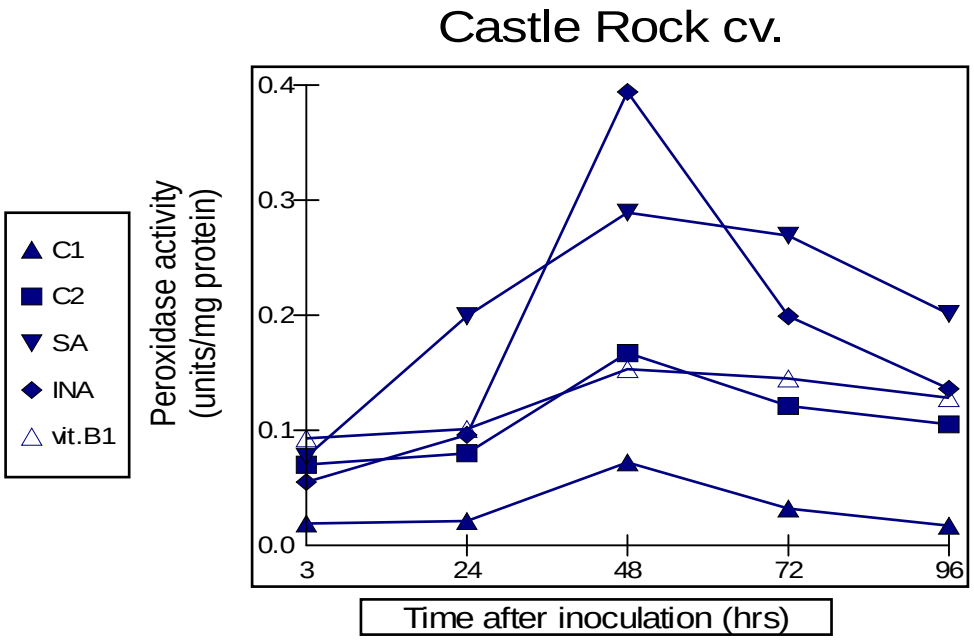

Fig. (7). Time course of peroxidase activities in early blight susceptible tomato cv., pre-treated with the tested chemical SAR inducers and inoculated with A.solani, at different periods following inoculation. C1 = untreated non-inoculated control, C2 = untreated inoculated control.

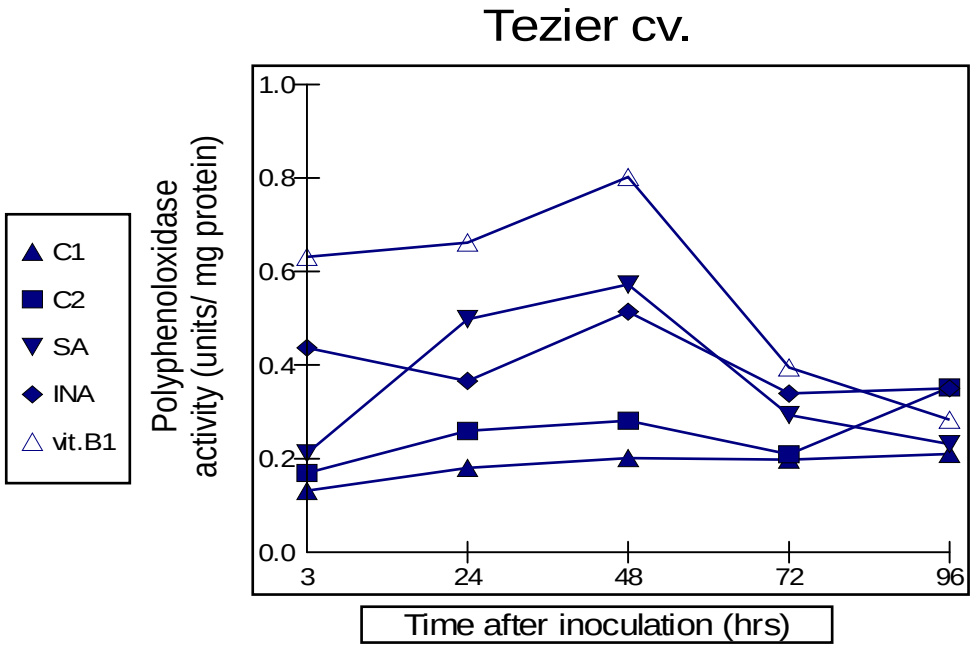

Fig. (8). Time course of polypenoloxidase activities in early blight resistant tomato cv., pre-treated with the tested chemical SAR inducers and inoculated with *A.solani*, at different periods following inoculation. C1 = untreated non-inoculated control, C2 = untreated inoculated control.

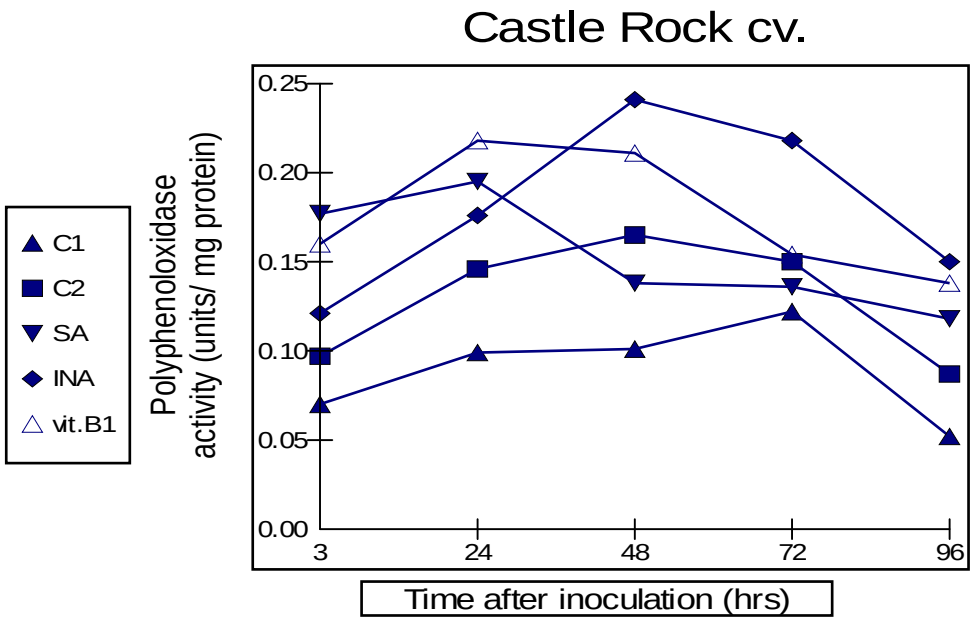

Fig. (9). Time course of polypenoloxidase activities in early blight susceptible tomato cv., pre-treated with the tested chemical SAR inducers and inoculated with *A.solani*, at different periods following inoculation. C1 = untreated non-inoculated control, C2 = untreated inoculated control.

Because SA is an important signal molecule (Enkerli *et al.* 1998 and Enyedi *et al.* 1992), its level may increase endogenously prior to the activation of SAR in each of the host-pathogen interactions described above (Malamy *et al.* 1990; Metraux *et al.* 1990). In the current study, the acquisition of systemic resistance to *A. solani* in tomato tightly correlated with elevated endogenous SA levels and the expression of the PRs genes. The minimum level of SA that is required to activate SAR in tobacco is approximately 0.33 µg g^{-1} FW (Yalpani *et al.* 1991) this conflict with our observation of SAR induction in tomato at foliar levels of 0.01 µg g^{-1} FW. and were unable to induce the expression of SAR. Following pathogen infection, increased levels of SA are known to occur (Enyedi, *et al.* 1992; Malamay *et al.* 1990 and Metraux *et al.* 1990). In our findings there was a fluctuation in endogenous SA levels of pretreated plants with chemical inducers then inoculated with pathogen *A. solani*) between the various treatments. Spletzer and Enyedi (1999) wondered that it was unclear why SA levels should fluctuate following plant pathogen infection from initial elevated SA levels. Plants that did not receive the chemical inducers exhibited endogenous SA levels following infection by *A. solani* and surpassed some inducers treatment levels in many cases either in resistant or susceptible cvs. It might be presumably, during early and later stages, pathogen serve as SAR inducer.

Regardless of the chemical inducers concentration used in PDA, there was no significant decrease in the radial growth of *A.solani* mycelium (not published). This indicates that SA does not have a direct antifungal effect on *A. solani*, but rather serves to trigger the signal transduction pathway that ultimately gives rise to SAR (Hunt and Ryals, 1996 and El-Mougy, 2002). Chemical-induced resistance to *A. solani* is likely due to the elicitation of a set plant defense responses. PR proteins (Vernooij *et al.* 1995) are considered to constitute one important portion of the induced defense responses employed by SAR (Enyedi *et al.* 1992; Hunt and Ryals, 1996; Van Kan *et al.* 1995). In spite of many reports approved that INA activates components of the SAR signaling pathway downstream of SA accumulation (Vernooij *et al.* 1995; Dong and Beer, 2000), our results demonstrated that there is considerable levels of endogenous SA accumulation. Although these levels were higher against other treatments but it continued greater significantly in resistant cv. (Tezier) than in susceptible (Castle Rock).

The development of SAR is associated with the induction of pathogenesis related (*PR*) gene expression. Increases in the endogenous SA levels in the pathogen-inoculated plants coincide with the increased levels of the *PR* gene expression and enhanced disease resistance (Sandhu *et al.* 2009) and plants are able to coordinate the expression of specific

PR genes in response to attack by relevant pathogens at the molecular level (Zhang et al. 2010). Several mechanisms that mediate the disease protection induced by certain chemicals have been described, including the direct inhibition of pathogen growth, blocking of the disease cycle (Fabritius *et al.*, 1997; Thompson *et al.*, 2000; Vicentini *et al.*, 2002), and the induction of plant resistance to pathogen infection (Dong and Beer, 2000; Nakashita *et al.*, 2003). Given the disease-progress-inhibiting activities of thiamine (vit. B_1) and riboflavin (vit. B_2) against fungal, bacterial, and viral pathogens, it would be unusual if these compounds acted as specific antibiotics. Media containing thiamine (vit.B_1, SA, and INA, did not inhibit the growth of *A. solani* on plates (data was done by authors in separate work). These results imply that these chemicals induce resistance in plants to infection by various pathogens. Broad-spectrum effects and the absence of direct effects on the pathogen are distinctive characteristics of other plant defense activators, including DCINA (Delaney, 1997), probenazole (Midoh and Iwata, 1996), probenazole derivatives (Yoshioka *et al.*, 2001), and brassinolide (Nakashita *et al.*, 2003).

Thiamine confers disease resistance through the priming of several plant defense responses, leading to a restriction of pathogen growth in planta and suppressed propagation of the inoculums. The maintenance of the resistance mimic status for a long period indicates that thiamine is a good candidate as a plant defense activation agent (Ahn, *et al.*, 2005). In addition, thiamine did not result in phytotoxicity at any of the tested concentrations. These results show that thiamine satisfies the requisites for an activator of plant SAR (Friedrich *et al.*, 1996), as previously suggested. Our results demonstrated that the higher accumulation levels of PRs due to exogenous application of thiamin (vit. B_1) against *A.solani* began at early stage after pathogen inoculation about 1-2 days, that's agreed with some suggestions of (Ahn, *et al.*, 2005) who found that the transcripts of all of the tested defense-related genes accumulated within 24 h after thiamine treatment, but the high transcript levels did not persist. However, following pathogen infection, SAR-related proteins were rapidly and strongly expressed in thiamine-treated plants, mirroring the expression patterns that occur during the interaction between resistant and susceptible host plants and pathogens.

Formulations of INA have been shown to be effective in decreasing susceptibility to pathogens and inducing PR proteins in several other plant species (Metraux *et al.*, 1991; Hijwegen and Verhaar, 1994; Kogel *et al.*, 1994; Nielsen *et al.*, 1994; Dann and Deverall, 1995; Vernooij *et al.*, 1995). According to colorimetric analysis in the present study, INA exhibited significantly higher levels in accumulation of *β*-1, 3-glucanase and chitinase in "Tezier" at 2nd day, peroxidase in resistant and susceptible to *A. solani* in the 2nd day and polyphenol oxidase in "Tezier" at the 2nd day and in "Castle rock" against *A. solani* in the 2nd - 4th days, that's confirmed the capability of this compound in induction of quantitative amounts of defensive proteins limits the progress of pathogen, this similar to Van Kan *et al.* (1995) whom they found that the spray of tomato leaves with INA formulation material alone apparently induced mRNAs for two PR proteins (osmotin and PR-4). INA is interpreted to act by moving rapidly into and systemically through plants, as shown by Metraux *et al.* (1991) using a radioactive form. The subsequent increases in the PR protein, chitinase, and in levels of resistance in parts of the plant remote from the point of application were concluded to be consequences of the accumulation of INA in these parts. This conclusion is supported here by the action of INA alone in raising activities of *β*-1, 3-glucanase, chitinase and peroxidase and resistance to early blight, in tomato seedlings, when contrasted with the effects of a control (H_2O) and untreated plants (pathogen only).

Salicylic acid (SA) played an important role in plant defense by the development of a systemic acquired resistance against pathogens (Ryals *et al.*, 1994) and by increasing antioxidant enzymes (Janda *et al.*, 1999). Exogenous applications of SA, either by direct injection or by spraying, have been reported to cause a multitude of effects on the morphology and physiology of plants (Pancheva *et al.*, 1996; Peng *et al.* 2004). In this series of experiments, we demonstrated that SA can activate a form of systemic resistance through accumulate a significant quantities of PR proteins against *A. solani* in greenhouse grown tomato plants. This was accomplished by providing 500 ppm SA directly to the shoot system of the plant, although Van Kan *et al.* (1995) reported that foliar application of SA to tomato has been attempted, but is an ineffective method for the introduction of SA to the leaf and did not result in accumulation of PR proteins. When we analyzed the PRs activity calorimetrically following SA treatment, we found the higher expressed levels of both, β-1, 3-glucanase and peroxidase in resistant "Tezier" and susceptible "Castle rock" at 48-h time point, but for polyphenol oxidase was at both 24- and 48-h time points, while for chitinase levels the increase was maintained throughout over the period of study up to 4th day. Induction of PR gene expression following SA application has also been demonstrated in tobacco, Arabidopsis, and cucumber plants (Metraux *et al.* 1990; Ward *et al.* 1991). Van Kan *et al.* (1995) report a similar finding after 24 h for an extracellular PR-1 transcript (P6) following SA feeding of an excised tomato leaf; however, this particular study made no

distinction between SA, and other inducers (vit.B_1, and INA) in PRs induction.

5. Conclusion

We have found that the accumulation patterns of chitinase, β-1,3-glucanase, peroxidase and polyphenol oxidase varies between early blight resistant cultivar (Tezier) and the highly susceptible variety, Castle rock. The activities of these defense enzymes were more in the compatible than the incompatible interaction of tomato with *A. solani*, suggesting that induction of these enzymes may be significant in symptom development in tomato shoots. That's supported by the higher endogenous levels of SA in resistant cv. compared to susceptible one, which the strong marker of systemic acquired resistance. In further investigations we plan to evaluate the effectiveness of chemical inducers in the field on some plant pathogens.

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